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VIRUS DETECTION: LIMITS AND STRATEGIES

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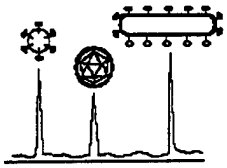

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The rapid detection of virus particles is a vexing issue to the military and public health communities. The need for a rapid and trustworthy virus detector remains an urgent and continuing requirement for commanders requiring an assurance that they have early warning from these threats. This short report presents a historical summary, with references, of methods used to extract, purify, and concentrate viruses. Working with viruses is not an easy job. The requirement for detection was identified nearly 60 years ago and just now is seeing an approach with the evolution of the Integrated Virus Detector System (IVDS) device to undertake this exacting and difficult task. This work expounds a new methodology - IVDS. The IVDS utilizes a physical process to detect viruses. This is a departure from the traditional approaches that historically have utilized obscure chemical reactions, complicated reagents and other exacting and difficult procedures for detection. The IVDS is based in four physical stages which are collection, separation, purification, and detection. Development and risks are discussed. An IVDS development schedule and costs are given that could lead to an advance prototype in two years. The IVDS system promises a very sensitive, broad spectrum "generic" virus detector free from reagent based reactions, simple to operate, and providing a capability for continuous monitoring and recording of virus levels in the outdoor bioaerosol.

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PREFACE

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LIMITS AND STRATEGIES

1. INTRODUCTION

In a very real sense, viruses have already demonstrated their effectiveness as weapons of warfare through a number of historic events. The pandemic, during W.W.I, of Spanish flu killed approximately 20 million people and is considered by many to have been a primary factor in the defeat of the German Army. According to the virologist Robert Webster (Ref.1), "It wasn't the Americans coming to Europe, it was the virus they brought that did the job." Similarly, epidemics caused by pathogens, particularly smallpox, brought from Europe to America wiped out nearly 90% of the American Indian population, and there is clear evidence that smallpox was used deliberately as a weapon (Ref.2).

In 430 B.C., sequestering of the Athenian population behind walls as a defensive move during the Peloponnesian war caused a great epidemic, probably smallpox or a virulent strain of measles (Ref.3). Armies of ancient Greece, and later those of Rome and Persia were know to poison the drinking water of their enemies with diseased remains (Ref.4). Returning to more recent developments, viral agent candidates that were tested by the Japanese biological warfare program in the 1930s and 1940s included yellow fever, hepatitis, encephalitis and hemorrhagic fever (Ref.5). Most recently, it has been suggested that prolonging Desert Storm or the Panamanian actions by even a few weeks could have exposed U.S. troops to serious threats of Crimean-Congo hemorrhagic fever or Sandfly fever viruses, respectively (Ref.6).

In this paper the problem of monitoring viruses is reviewed, particularly airborne viruses, from the perspective of an someone who wants to know: "Faced with the inherent problems, which methodologies will actually work and provide workable practical solutions?" The solution calls for semi-quantitative analyses of background interference, sensitivities, specificities, tolerances, costs and design specifications. The approach must differ fundamentally from the past where people typically espoused either a particular method (often in a highly idealized and thus irrelevant setting, such as a clean room, etc. which did not consider the outdoor bioaersol issues or the contamination associated with a battlefield), or more general, qualitative issues surrounding viral warfare.

2. STATEMENT OF THE PROBLEM

There are many inherent challenges in airborne virus detection. From the standpoint of defense against military and terrorist threats - and the more global perspective of public health - the detection of airborne viruses is in particular a technological challenge that makes chemical warfare (CW) detection issues look easy in comparison (Ref.7). Factors contributing to the extraordinary difficulty of the task include the wide range of viruses carried in the air, the exceedingly large background of biological components relative to infectious levels of viruses, and the problem of mutation, either by natural or artificial means, which greatly enlarges the inventory of potential BW viral weaponry.

Purification and concentration from the background material is required whatever the detection method to be used in subsequent steps. Background loading of biological samples is astronomically large, and the virus materials of interest must be separated from the biological debris. A five million fold concentration factor is required, whether the next step is based on biological activity or physical attributes. There is no purpose in even considering the detection of viruses until the purification and concentration problems are resolved.

3. HISTORIC METHODS

There are historic methods for the purification, extraction, and concentration of viruses which will be discussed here. For more than sixty years scientists have tried methods to isolate and identify viruses. It has continued to be a complex research area, with many thorny problems associated with what man has wanted to do versus what nature has allowed. Viruses, by their very nature of being among the smaller "living" organisms, and their ability to mutate and change almost at will, have confounded the search. Historically speaking, we have made inroads into this problem, but before the present IVDS system little real progress has been made in the sampling and counting of airborne viruses.

Many methods have been tried over the years to extract, purify and concentrate viruses for analysis. Table 1 gives a summary of the various techniques and the seminal reference for each of these methods. Nearly all the methods have major shortfalls either in selectivity or their application to all viruses, or in their recovery. Of the 18 familiar process presented, only two have a high selectivity, are universal for all viruses and are excellent for recovery. The other 16 processes have inherent and limiting weaknesses which are fundamental to their processes which limit their technologies to virus detection. Some of these limitations may never see a

solution regardless of how long and determinedly we work on them. A successful methodology should make use of the processes that have achieved high marks in all three categories. The IVDS device makes use of the physical methods that have high marks in all three areas. The physical properties are not dependent upon complex reactions and thus are a reliable basis for building a virus detection methodology.

4. INTEGRATED VIRUS DETECTION SYSTEM (IVDS)

A new system for detecting and analyzing viruses was proposed in 1993 which utilized advances in historically successful technologies combined with new advances in filtration technologies and computers (Ref.8). The IVDS device builds upon the successful sedimentation rate and density gradient centrifugation technologies which date from the early 1930s and have since that time reached a high degree of advancement. Combined with a new ultrafiltration system and well-understood Differential Mobility Analyzer and Condensation Nucleus Counter technologies it was possible to build a new system for extracting/purification and counting viruses. This new device was developed in four distinct stages which are integrated together to form the IVDS. The four stages are: 1) Collection Stage, 2) Extraction Stage, 3) Concentration Stage and 4) the Detection Stage.

The various capabilities, and comments concerning these capabilities, are given in Table 2. The four stages, their associated technologies, advantages and risks are summarized in Table 3. Because the IVDS system makes use of the fundamental physical properties associated with viruses much of the risk historically associated with virus detection is minimized. Since the IVDS device is utilizing well known and highly successful commercial equipment there is a minimal or low risk in the instrumentation associated with IVDS. The only risks, which are minor, are the integration of the some of the parts into an advanced prototype. This integration step follows many similar and successful examples and does not appear to present any unusual difficulties.

4.1 Collection Stage

Sample collection will use the Army's XM2/XM19 collector. It is an established system and until a new method is introduced it will serve to at least approach the target specifications of the IVDS system. It should be noted that the IVDS will count the virus particles for many kinds of liquid samples from any source. The initial use of the XM2/XM19 avoids the need to develop new technology and it is already used as an integral part of the BIDS system. The sample volume needed for

Table 1. Methods to Extract, Purify, and Concentrate Viruses for Analysis

A graphic summary of methods for purification/extraction/concentration of viruses, giving: the seminal reference, and the time period during which the method developed into a routine technique for virus purification; the selectivity/specificity for viruses, qualitatively; whether or not the method applies to all viruses of human pathogenicity; and how well the method performs if the viruses are adsorbed to suspended solids.

Virus purification method	First developed for routine separation of viruses (and seminal ref.)	Selectivity for viruses	Universal for all viruses?	Recovery of solids-associated viruses
Sedimentation rate centrifugation	1930's (1:1922; 2:1931)	High when both	Yes	Excellent
Density gradient centrifugation	Mid-1950's (3:1951)	centrifuge methods used	Yes	Excellent
Ultrafiltration	1940's (4:1933)	Low	Yes	Poor
Virus-adsorbent filters	Mid-1970's (5:1972)	Low	No (pH)	Poor
Size-exclusion chromatography	1960's (6:1962)	Moderate	Yes	Poor
Antibody-based chromatography	Late-1950's (7:1954)	High	No	Moderate
Ion-exchange chromatography	Late-1950's (8:1952)	Moderate	No	Moderate
Adsorption chromatography	1960's (9:1958)	Low	No (pH)	Moderate
Immobilized receptors	Never (10:1978)	Moderate	No	Moderate
PEG hydroextraction-dialysis	Never (11:1959)	Low	Yes	Good
Organic/inorganic flocculation	1970's (12:1941; 13:1971)	Moderate	No (pH)	Poor
Two-phase liquid-liquid separations	1960's (14:1959)	Low	Yes	Good
Polymer precipitation	1960's (15:1942)	Low	Yes	Poor
Adsorption to cells and particles	1940's (16:1931)	Moderate	No	Moderate
Electrophoresis	1950's (17:1947)	High	Yes	Poor
DNA/RNA Hybridization	1970's (18:1965)	High	Yes	N/A
Crystallization of viruses	Never (19:1949)	High	No	Poor
Enzyme digestion	Never (20:1954)	Moderate	No	Good

Methods that require the use of low- or high-pH buffers (e.g., to charge virus particles) are not considered universal, since many viruses are disrupted by such buffers; these are indicated by "No (pH)".
Methods that only remove specific impurities are not included.

1. MacCallum, W.G. and Oppenheimer, E.H. (1922) J. Am. Med. Assoc. 78:410.

Table 2. General Features: Integrated Virus Detection System (IVDS).

Capability	Comments
Extraordinary sensitivity.	10 virus particles/liter of air. (The Joint Program Office requirement is to detect 20,000 pfu/liter. Each PFU can contain many virus particles.)
Freedom from false negatives, i.e., broad spectrum detection.	The system must accommodate the high mutation rates of known viruses, i.e., must still be able to detect them after mutation.
Freedom from false positives associated background.	Background loading will always be astronomically larger than the amount of virus collected; thus, exquisite purification is necessary; a five million concentration is needed, whatever the detection method that is ultimately used.
Rapid detection.	The objective is for a total processing time (after collection) of less than 15 minutes at the 10 viruses/liter of air threshold.
Limited need for for biochemical reagents.	These reagents are difficult even for trained virologists to use. They also increase the incidence of false positives several hundred fold, even under ideal conditions.
Engineerable for field use.	The first generation IVDS will have a volume of about 36 cu ft and cost less than $200,000 (unit procurement cost).

the IVDS is small, about 10ul/minute. This amount should be able to be split from the sample stream of the XM2 without effect on the other components of the BIDS.

The development and insertion of the IVDS matches the overall philosophy and design approach of the BIDS system. The BIDS system already exists, though its composition is constantly changing. The approach has been to field a system of non-developmental components, then replace those components as improved capabilities become available. At such a time as the XM2 may be replaced the IVDS would simply adapt to the sample stream provided by the new system.

4.2 Separation Stage

The separation (extraction) stage (ultracentrifuge) starts with the viral containing material from the collection stage. It applies centrifugation technology, with original applications more than 70 years old, demonstrated at Oak Ridge National Labs (ORNL) in the 1940s. Initial requirements at ORNL called for the separation of nuclear fuel material. Over time it was improved and applied to other uses.

This technology, known as ultracentrifugation, has over the last many years become highly developed. This application to viruses represents a renewed use. Patent applications were filed in 1996 which cover the following principal ideas for using this technology for separating viruses.

The ultracentrifugation process for IVDS takes advantage of the *Virus Window*, a phenomenon in which virus particles are physically separated from other material and is based on specific physical properties. The virus groups are in reality separated into a 3-Dimensional address. This address is unique for the various virus families and most likely for the viruses within the families.

In the 1960s engineers at ORNL perfected the centrifuge technology designed specifically to extract viruses from the biological background. They expounded the *Virus Window* as the area bounded by density of 1.175 and 1.5 gm/ml and sedimentation coefficients of 100 and 10,000 Svedberg units. This window represents a real 3-D physical location of the virus material within the centrifuge.

Ultrcentrifugation offers several advantages. It provides universal capture of all viruses, with proven capture efficiencies better than 95%. This is very important since we will necessarily be dealing with very small quantities of material. UF technology permits recovery approaching 100% at flow rates of up to 320 ml per minute. The flow rates used in IVDS are expected to be lower, as a result high recovery rates, approaching 100%, will be routine.

Ultracentrifugation also provides a high degree of physical separation from background components and does not depend upon biochemical reagents, in which dependence is bad and leads to all the difficult and often complex techniques rift and prone for error. Reducing the need for reagents has several immediate advantages. The technical advantage is to avoid the need for highly skilled technical laboratory personnel. Even for them the historic processes using reagents are sensitive and error prone. Eliminating the reagents makes analysis easier and reduces the training required for a soldier operator.

Elimination of reagents improves the accuracy, and the rate of the output. It further reduces the logistics requirements of storing and resupplying consumable components, which is important on an active battlefield. This is a particularly attractive advantage considering the expense and monitoring requirements that accompany shelf life limited items.

There are 20 viral families that contain viruses pathogenic to humans. After plotting the 3-Dimensional addersses of the virus positions in the *window* there are two key conclusions: first, is that all of these viruses lie in an area free from interference from all other components or background material and second, they are separable, with surprisingly little overlap between the 3-Dimensional addresses. In other words, knowing the density and size of a detected virus particle pinpoints it to a particular virus family, in nearly every case.

During operation the centrifuge operates in a flow process. The stream of liquid from the collector stage flows continuously into the rotor assembly. The rotor assembly provides a spinning cylinder of a solution of specified density; as this cylinder of solution is rotated the material assumes a density gradient. The viruses separate according to their 3-D address. These isolations are sent along the detector in the following subsequent stages to be counted and since they are identified by their unique location they also can be identified. Thus the number and identity of the viruses in the sample are listed.

4.3 Purification Stage

Connecting this purification/concentration stage (ultrafiltration) with the previous extraction stage provides the unique edge of the IVDS. This stage provides the exquisite purification of the target viruses from an overwhelmingly high background of extraneous material.

After the ultracentrifugation extraction stage has separated the materials of interest by taking advantage of the *Virus Window*, the sample is purified and concentrated by using an

ultra filtration (UF) process. The UF selected uses a patented technology developed by Lyotropics Inc., which provides a solution to this problem.

The UF step accomplishes two tasks. It first separates viruses from soluble proteins and then concentrates them into a small volume of liquid.

A technological issue which has been solved by work at ERDEC in cooperation with Lyotropics is the issue of pore size of the UF material. This pore size is critical to the success of this process. The size range for viruses of interest is 22nm to 100nm. These viruses fall within the unique *Virus Window* observed during ultracentrifugation.

There are interfering proteins with sizes up to 20nm, so pore size control of the UF process (particularly at this point) is critical so that the 20nm particles pass through but he 22nm sized particles are retained. The material developed satisfies this requirement.

4.4 Detection Stage

The detection stage (ES-DMA-CNC, explained below) serves two purposes. First it counts the individual particles and second, it determines the size of the detected particles. When used with the ultracentrifuge stage the viruses are separated according to their unique 3-Dimensional addresses and the counter is then counting a specific group of viruses and in this manner they can be identified. When used without the ultracentrifuge stage. the detector stage counts all the virus particles present. With out the specific 3-Dimensional address from the ultracentrifuge stage no identification is possible without further steps. This is a physical reality of how viruses behave and in this manner represents a new way of detecting and counting these microorganisms.

The major components of the Detection Stage are an electrospray (ES) nozzle, differential mobility analyzer (DMA), and a condensation nucleus counter (CNC, or sometimes CPC for particle counter). TSI, Inc., based in St. Paul, MN, has established itself as a source for these components and the various integrated combinations. As with the other stages of IVDS, using components already in commercial use reduces the developmental risk. The ES and DMA capabilities are available commercially as a single integrated unit. The DMA and CNC capabilities are also used as an integrated unit.

Recent work has resulted in the ES being integrated with the DMA-CNC pair making the triplet combination ES-DMA-CNC. This product is used in the IVDS. This combination of current,

well tested, instrumentation provides a bold, physical method to detect virus. It is not dependent upon some obscure chemical reaction, or dependent upon the time for some reagent to interact, or dependent upon any other factor other than their inherent physical properties, which are unlikely to change, for detection and identification. This is a real change in the way of doing business in the detection of viruses and represents a process which for the first time in nearly 60 years solves the virus detection requirement.

Table 3. How IVDS Meets the Requirements

Capability	Comments
Extraordinary sensitivity.	Detects as few as 10 virus particles per liter of air.
Freedom from false negatives, i.e. broad spectrum detection.	Takes advantage of the *virus window*. All virus particles of interest lie within this region.
Freedom from false positives associated with background.	Ultracentrifugation followed by ultrafiltration provides a sample essentially free of extraneous particles. Dissolved material evaporates during the electrospray process and cannot act as a condensation nucleus in the counter.
Rapid Detection.	The process (after sample collection) will take approximently 15 minutes.
Limited need for biochemical reagents.	No reagents needed for biochemical processing other than very small amounts of butanol needed for detection.
Engineerable for field use.	Highly developed technologies should ease the engineering issues that arise to make the IVDS suitable for field use. The first generation IVDS will have a volume of about 36 cu ft and cost less than $200,000 (unit procurement cost).

5. IVDS DEVELOPMENT AND RISKS

5.1 Collection Stage

The XM2 collector is fully developed, and has been tested for virus collection. The XM2 samples airborne particles in the range of 2-10 microns at a collection rate of 1000 liters per minute. IVDS can utilize any new collector or simply can process a liquid that is directly inserted to count and identify the virus particles. The collection stage is considered a low risk.

5.2 Extraction Stage

The work completed by the Oak Ridge National Laboratory in the 1960s demonstrated a 95% or higher virus recovery at flow rates of 100 L/hour or more in times of less than 30 minutes. This successful work was not actively pursued because of the missing Ultra Filtration and computer technologies at the time. A further reason was a lack of a requirement for a virus detector at the time. Current requirements and urgent needs have changed this position, and the advancement of the various technologies used in the IVDS makes this process a low to moderate risk.

5.3 Purification/Concentration Stage

The ultrafiltration membranes and techniques developed by Lyotropics, Inc. permit exceptional control of pore size and a tangential flow process. This technology has also been demonstrated and only needs to be integrated into the IVDS. The integration is considered not difficult but could require precise manufacturing, and thus has a low to moderate risk.

5.4 Detection Stage

The combination of electrospray, differential mobility analyzer, and condensation nucleus counter is available from TSI Corporation. The technology has been developed and demonstrated for IVDS purpose. This is considered a low risk stage.

An explanation of the technologies and their associated risks are given in Table 4.

Table 4. Explanation of Technologies and Risks

Stage	Technology	Advantages	Risks
Collection Stage	Multiple Technologies	Standard Army Collectors, no development	Minor risk that rest of BIDS cannot spare sample needed for IVDS
Extraction Stage(*)	Ultracentrifugation	Highly developed technology; National center of expertise at ONRL	New application at this sample size
Concentration Stage(*)	Ultrafiltration	Commercially successful	Integrated into IVDS
Detection Stage(*)	Differential Mobility Analyzer and Condensation Nucleus Counter	Highly developed technology; available commercially as an integrated unit	Integration into IVDS

(*) Patents apply

6. IVDS SCHEDULE AND COSTS

Development of the IVDS is broken into four subprojects, each with a working device which can be demonstrated. It should be noted that some of these subprojects can be run in parallel to shorten the overall project to integrated system demonstration time. The following Table 5 summarizes these subprojects.

Table 5. IVDS Schedule and Cost

Sub-Project	Time and Cost	Comments
1. Construct ultra filtration (UF) virus purifier-concentrator (*)	18 weeks $115K	UF with 20nm poresize supported by micro-filter, in housing
2. Integrate UF purifier with electro-spray-condensation nucleus counter	15 weeks $95K	The exit port from #1 will be configured to serve as the electro-spray jet; this will feed directly into the CNC so the viruses can be counted
3. Centrifugation stage(*); first generation model	11 months $545K	Update proven 1960 capability emphasizing decreased run times and volumes, and matching the flow rates to the neighboring stages
4. Integrate components	6 months $350K	Provide a working prototype IVDS

(*) Patents apply

7. SUMMARY AND RECOMMENDATIONS

Many methods have been proposed and used historically for the separation and identification of viruses. All of these attempts have not been entirely successful and certainly not very helpful to military requirements that call for a quick analysis and assessment of a virus attack. The important issue has not been solved and a simple off-the-shelf solution does not look promising. A bold new concept based upon historically successful processes is in order and is suggested which uses a combination of technologies, integrated into a new system, called the Integrated Virus Detection System (IVDS). This device represents an enormous and fundamental breakthrough in virus detection. The complex issues associated with the historical approaches have been solved and the IVDS device can be expected to be the virus detection and characterization device of the future.

It is evident that a wide-ranged "generic" virus detector must be pursued. This is especially urgent given the variety of viruses, their numbers, their ease in mutation and their abundance in the background in relation to potential pathogenic forms and military or terrorism applications of these "sharks" of the microbial world. The entire aspect of the public health issues associated with the use of virus agents is not missed on the applications of a new device designed to count and classify viruses.

At this time, the IVDS technology promises our best hope for a virus detector system for the next twenty years. This device can be integrated into our current military systems and provide the capabilities to meet the urgent needs of the soldier in the field to the public health needs of the country.

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